EVALUATION OF NATURAL PRODUCTS AS POSSIBLE ALTERNATIVES TO METHYL BROMIDE IN SOIL FUMIGATION

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ABSTRACT

Crude extracts from *Warburgia ugandensis* Sprague, *Azadirachta indica*, *Tagetes minuta* and *Urtica massaica* have been tested against soil pathogens; *Fusarium oxysporum*, *Alternaria passiflorae* and *Aspergillus niger*. The results obtained showed biological activity against these pathogens except for *U. massaica* extract, which was not active. Separation of the crude extract from *W. ugandensis* yielded two pure compounds; Muzigadial and Muzigadiolide whose structures were confirmed by spectroscopic techniques and comparing with already existing spectroscopic data. The minimum inhibitory concentration (MIC) for those two compounds from *W. ugandensis* have been obtained. Green house experiments using *W. ugandensis* crude extract against *F. oxysporium* that causes wilting in *Lycopersicon esculentum* (tomato plant) have been carried out. Data interpretation carried out by Analysis of variance and comparison of various treatments through LSD and DMRT tests showed that this crude extract controlled the pathogen *F. oxysporum*.

INTRODUCTION

Methyl bromide has been used as a fumigant for over 60 years. An important valuable property of methyl bromide is the broad range of pests that it can control. It is used as a fumigant against pathogens (fungi, bacteria and soil borne viruses), insects, mites, nematodes and rodents. These pests may be in the soil, in durable or perishable commodities, as in structures and transportation vehicles. The broad spectrum of activity and ease of application of the material have led to its use as treatment of choice in a number of situations. Several agricultural systems involving intensive production of high value crops have become dependent on the use of methyl bromide. On a global basis, the largest single use of methyl bromide is as a soil fumigant (1).

Although methyl bromide is a most useful tool in specific instances, there are a number of technical and legislative limitations which have led to restrictions on its use. Methyl bromide can have adverse effects on a number of commodities, causing taint and odours. It also has a substantial phytotoxicity. Treatments with methyl bromide result in production of bromide ion residue. These may accumulate in excessive levels in commodities that are fumigated several times and have been a cause of concern in ground water in some European countries. Of most concern is its ozone depleting potential.

Methyl bromide was listed as an ozone depleting substance by the fourth meeting of the parties to the Montreal Protocol on Substances that Deplete the Ozone Layer in Copenhagen in November 1992. Due to this global problem, it is supposed to be phased out. The listing of the fumigant methyl bromide under the Montreal protocol is causing concern to plant health services throughout the world. No other treatment matches methyl bromide fumigation for wide ranging efficacy, reliability and speed of action. The methyl bromide technical options committee (MBTOC) established by the parties to the protocol to review technical issues concerning methyl bromide did address the technical availability of chemical and non-chemical alternatives for the current uses of methyl bromide (1). For soil treatment, non-chemical alternatives to methyl bromide include cultural practices, biological control, organic amendments and physical methods. Possible chemical alternatives can either be fumigants or non-fumigants. Available fumigant chemical alternatives include methyl isothiocyanate (MITC), MITC generators, metasodium and dazomet. Halogenated hydrocarbons are other available fumigant chemical alternatives. These include 1,3-dichloropropene (1,3-D), chloropicrin (trichloronitromethane) and ethylene dibromide (EDB). All these chemicals have setbacks such as phytotoxicity, skin and eye iritations, sensitisers, genotoxins and carcinogens.

All non-fumigant nematicides are organophosphates or carbamates and therefore are highly acutely toxic neurotoxins (cholinesterase inhibitors). None of the available chemical alternatives alone offers broad-spectrum disinfestation attributes of methyl bromide. Non fumigant alternatives are especially problematic due to ability of soil pests to develop resistance or the potential of soil microflora to decompose these chemicals (1).

Plants under study

It is a fact that natural products from plants remain a vastly underutilised resource for the discovery of novel antimicrobial compounds yet we live in a world where most pathogens can be controlled by these natural products. The majority of higher plant species are yet to be explored as potential sources of antimicrobial agents. The use of botanicals in disease management has been going on for a very long time in traditional practices. Currently, organic farming which aims at healthy crop production without usage of chemicals, but by incorporation of natural weeds and plants in the farms that effectively aid in pest control, is being practiced in some places in Kenya. It is from traditional practices, organic farming and some documented cases that this work was based.

The plants under study include *W. ugandensis*, *Azadrachta indica* (neem tree), *T. minuta* (Mexican merigold), and *U. massaica* Mildbr. (Stinging nettle).

The genus *Warburgia* (Conellaceae) consists of two species distributed in East Africa, *W. stuhlmanii* Engl. and *W. ugandensis*. The two species are widely used in the local folk medicine to alleviate toothache, neumatism, general body pains, diarrhoea and malaria. In addition, the leaves of *W. ugandensis* are sometimes used locally as a spice for food. The bark of *W. ugandensis* is commonly known by several different names depending on the local tribe such as 'Apacha' (Luhya), 'Muthiga' (Kikuyu), 'Olosogoni' (Maasai), 'Soget' (Kipsigis), 'Soke' (Tugen) and 'Sogo-maitha' (Luo). The distribution of *W. stuhlmanii* is limited to the coastal areas, and is known as Mukaa (Swahili)(2).

The aqueous methanolic extracts of the barks of *W. ugandensis* and *W. stuhlmanii* are active against gram-positive bacteria, yeast and filamentous fungi. A series of unique sesquiterpentine 1-4 dialdehydes isolated from these plants have been shown to have broad antibacterial and antifungal activities. Polygodial, Warbuganal and muzigadial obtained from these plants show similar antibacterial spectra (3). These dialdehydes possess potent antifeedant activity against African armyworms (4). One more stricking feature of these molecules is their hot taste for the human tongue, which parallels their feeding inhibition of animals. These properties however are related the stereochemistry of the C-9 aldehyde group (5). Detailed separation work has been done obtaining the 1-4 dialdehydes and other compounds. These compounds include polygodial, warbuganal,

muzigadial, ugandendial, mukaadial, cinnamolide, cinnamolide-3β-ol, cinnamolide-3-β-acetate, ugandensolide, deacetylugandensolide and muzigadiolide.

Azadrachta indica (Neem tree) is a member of the mahogany family Meliaceae. It is commonly known in swahili as mwarubaini (Muarobaini) (6). Neem contains a group of compounds called 'triterpenes', more specifically 'limonoids'. Azadirachtin, salanin, melantiol and nimbin are the best known. Azadirachtin has proved to be the tree's main agent for battling insects. It repels and disrupts growth and reproduction (it does not kill insects immediately). The tree has been known for its insect antifeedant properties (7). Nimbin and nimbidin have been found to have antiviral activity. Nimbidin is the primary component of the bitter principles obtained when neem seeds are extracted with alcohol (6). Apart from insects, neem affects quite a range of other organisms. These include nematodes, snails, crustaceans, and fungi (6), (8). In the world of medicinals, since antiquity, neem has been reknowned for healing. Almost every part of the tree has been used for the treatment of a variety of human ailments particularly against diseases of bacterial and fungal origin. In the world of human medicine, it has been used as a fungicide, antibacterial, and aniviral agents, for dermatological infections, dental treatments, chaga's disease, malaria, pain relief and fever reduction and birth control. In the field of veterinary medicine, it is used in controlling insects, bacteria and intestinal worms.

Tagetes minuta, a common weed, is in the family Compositae (Asteraceae) with several other species. It is an erect strong smelling annual, often very robust but variable in plant habit and very plastic in its response to crowding. Its leaves are pinnate with ellyptic toothed leaflets, heads are creamy yellow, in terminal corymbs with phyllaries 10 mm long (9). There have been reports on the use of *Tagetes* species as a nematicide. An example is the use of natural thiophene derivative from the roots of *T. jalisciencis* as a nematicide against *Melodigyne incognita* in light (10).

Urtica massaica (stinging nettle) is in the nettle family Urticaceae. It’s a very irritating, painful stinger often growing in abandoned tracts in the montane forest areas (9). It is mentioned as being active against fungal disease in traditional practices.

Test microorganisms

Many soil-borne pathogens are destructive parasites whose existence needs to be controlled where crop production is concerned. Fungi are among the soil borne microorganisms. Some fungal species are parasitic and destructive to host plants.

The test microorganisms under study belong to the species *Fusarium*, *Alternaria* and *Aspergillus*. *Fusarrium* is a large genus and of great interest as plant pathogens, causing seedling diseases of cereals, root rot, and wilt of several plants of economic importance. Many of the species are destructive parasites, invading the ducts of plants and by stoppage of water supply causing the class of diseases known as 'wilts'. Taken as a whole, the genus is one of the most injurious with which plant pathology has to do. *Fusarium* diseases are found in a number of plants; cereals and grasses, legumes and horticultural crops (11), (12). The genus *Fusarium* is a very successful 'soil inhabitant' and once established persists for years, rendering the soil unfit for profitable crop production.

EXPERIMENTAL

COLLECTION AND EXTRACTION OF PLANT MATERIAL

The plant leaves of *Warburgia ugandensis* were collected at Moi University, Forestry farm and nursery in September 1997. Its stem-bark was collected in Kerio Valley, Keiyo District, Kenya in May 1998. Plant leaves of *Azadirachta indica* were collected from Mombasa, Kenya in August 1997. The aerial parts of *Tagetes minuta* were collected around Chepkoilel Campus, Moi University. *Urtica massaica* leaves were collected at Molo, Nakuru district, Kenya. The herbarium of Department of Botany, Moi University authenticated the plant materials, and stored the voucher specimens.

Ground plant material was cold extracted with methanol and then filtered. The resultant solution was concentrated at reduced pressure using Böchi Rotary Vapour at a set temperature water bath of 40^{o}C. For the stem bark of *Warburgia ugandensis,* wet plant material, (3kg), was cold extracted with methanol. This resulting extract was concentrated to remove the methanol using the rotary vapour at 40^{o}C. The resulting extract was partitioned between water and chloroform. The chloroform fraction was concentrated to yield a crude extract weighing 95g.

SEPARATION

This was done for *W. ugandensis* bark extract. A portion of the concentrated chloroform fraction (15g) was filtered through TLC grade Merck silica gel using ethylacetate (EtOAc) under vacuum pressure. The filtered crude was concentrated. This crude was then packed in a prepacked Merck silica gel column (40-63mm), and separation achieved by flash chromatography, eluting with neat hexane first, and then hexane containing increasing amounts of EtOAc. The elution progress was monitored by TLC using 30% EtOAc in hexane as the developing solvent. Visualization of the chromatogram was achieved by UV lamp and concentrated sulphuric acid charring on a hot plate.

The crude of *W. ugandensis* leaves (20g) was fractionated over Merck silica gel (70-230 mesh) by normal column chromatography eluting with hexane and then hexane containing increasing amounts of acetone. The eluate fractions were tested in vitro against the test microorganisms.

From the bark, two compounds were obtained. The compounds were purified through crystallization using hexane.

STRUCTURE DETERMINATION

The IR data of the two compounds was obtained from their spectra run using Shimagzu-IR408 spectrophotometer. NMR spectra of the two compounds were obtained through measurements on Bruker ARX300 spectrometer that generated 300MHz NMR spectra operating at 300MHz (^{1}H) in $CDCl_3$ with TMS as internal standard.

Complete structure determination was achieved by comparing the IR and NMR data obtained with that in literature (13). The melting point data was obtained by use of Reichet Thermovar apparatus. This data was used to compare with that in literature. These two compounds were also tested against the test microorganisms and the Minimum Inhibitory Concentration was obtained.

BIOASSAYS

For the crude extracts, the diffusion method (14) was used to assess the antifungal activity against *Fusarium oxysporum, Alternaria passiflorae* and *Aspergillus niger.* Serial

concentrations of the crude were prepared so that 20µL of 30 % methanol in water contained 1mg, 10mg, 20mg, 50mg, 100mg, and 150mg of the crude for each plant extract. The fractionated *W. ugandensis* leaves extract was also tested. Filter paper discs were prepared, 12mm, in diameter, and loaded with the measured volume of the test extract solution (each prepared in triplicate) and kept in a dust free condition. The medium (2% malt extract: agar agar) was prepared by adding 20g of each in 1litre distilled water and heating to dissolve the contents. The media, distilled water and petri dishes were sterilized by autoclaving at 120^{o}C at 1 bar pressure for 20 minutes. A small amount of distilled sterilized water was used to dissolve the test microorganism. This was introduced to the media after cooling to a temperature just before it solidified and inverted several times to evenly distribute the spores. The media was then poured into the sterile petri dishes and allowed to solidify. The air-dried discs were then applied on the medium in the petri dishes and incubated for three days. The clear zone of growth inhibition around the disc was then measured and expressed as inhibition diameter.

Determination of MIC

This was done for the pure compounds. The agar macrodillution method (14) was used to determine the MIC. Both 2% malt extract agar: agar agar and Potato Dextrose Agar (PDA) media were used. Malt extract agar: agar agar medium was prepared as described in the diffusion method. PDA medium was prepared by suspending 39g in 1 litre of distilled water and heated to dissolve completely. The medium was sterilized by autoclaving at 120^{o}C for 20 minutes. Serial concentrations of the pure compounds, 3µg/ml, 5µg/ml, 10µg/ml, 12µg/ml, 25µg/ml, 50µg/ml and 100µg/ml were prepared in 20% ethanol-water solution, each in triplicate. This was incorporated into a liquefied agar medium (45-50^{o}C in a water bath) in sterile screw-capped tubes. The medium was mixed gently by inverting the tubes several times and the contents poured into an appropriate number of petri dishes. The plates were then set aside on a horizontal surface and allowed to solidify. One control plate containing the medium without any compound was prepared for each series of dilutions. Each plate was then incubated at 37^{o}C in an inverted position for three days. The lowest concentration at which no growth was observed visually was determined and indicated as the MIC.

GREEN HOUSE EXPERIMENTS

Culturing of the pathogen

The pathogen, *Fusarium oxysporum* was cultured in the laboratory two weeks before the field experiment set up. Malt extract agar: agar agar medium (500ml) was prepared by autoclaving at 120^{o}C at 1 bar pressure for 20 minutes. The spores of the pathogen were dissolved in a small amount of sterilized distilled water and then introduced into the prepared culture medium. This sterilized medium was inverted several times and shaken to evenly distribute the spores. It was then poured into 20 sterilized petri dishes, each containing approximately 25ml of the culture medium. The dishes were incubated at 37^{o}C in an inverted position.

Preparation of soils and pots

The soils were collected from a fertile field and pasteurized with aerated steam for sterilization before use. The pots were sterilized by cleaning with distilled water and rinsing with sodium hypochlorite and allowed to air dry. For soil infestation, the pathogen (*F. oxysporum*) spores, already cultured, were dissolved into 360ml distilled sterilized water. Then 10ml of this *Fusarium* inoculum solution stock ware taken and diluted to 100ml to make a total of 36 of such dilutions. This was poured over the surface of the soil in each pot and allowed to infiltrate the soil. Four pots did not receive this treatment.

The soils in the pots were allowed to stand in the greenhouse for a period of two weeks with occasional watering with distilled water before the introduction of the crude extract to allow the establishment of pathogen into the soil. A total of 10 treatments were established. Different crude concentrations (100ml each) were prepared; 1mg/ml, 2mg/ml, 5mg/ml, 7mg/ml, 10mg/ml, 12mg/ml, and 15mg/ml in 20% ethanol/water mixture in four replicates and poured on the surface of the soil. Four pots had 100ml of the solvent (20% ethanol/water) poured on the surface of the soil in each pot with no crude extract in it. This served as a negative control and helped in checking whether the solvent had any effect on the pathogen. Four more pots remained with the pathogen treatment without any treatment with the crude extract or the solvent. This served as a negative control. The four pots with no pathogen inoculum did not receive the crude extract and the solvent treatment. This served as the positive control. After this procedure, the soils were allowed a period of two weeks with occasional watering before planting.

Planting

Lycopersicon esculentum (certified moneymaker tomato) seeds were planted 2cm deep into the soil. Each pot had five seeds planted in it in all the four replicates for the ten treatments.

Experimental design

The experimental design was complete randomized block design, replicated four times. The pots were arranged randomly at a distance of 60cm from each other horizontally and at a distance of 30cm from each other within each block.

Data collection

For the fungal disease infection (*Fusarium* wilt) the parameter observed was the severity of infection of leaves indicated by wilted leaves, vascular discoloration of the hypocotyl tissues, branches and leaves exhibiting wilting and chlorosis, necrosis, premature defoliation and eventual plant death.

Data indicating the severity of infection was collected for a period of one month after seedling emergence. Disease scores for leaf infection were taken for 21, 23, 25, 27, 30, 35, 40, and 50 days after planting (DAP), after which the scoring remained consistent with the last day of scoring. This scoring was done according to a 1–9 scale (15):

Evaluation stages:

Scale	Disease Symptoms
1	No visible disease symptoms.
3	Very few wilted leaves (1-3 leaves representing no more than 10% of the foliage) combined with limited discoloration of the root hypocotyl tissues.
5	Approximately 25% of the leaves and the branches exhibit wilting and chlorosis.
7	Approximately 50% of the leaves and branches exhibit wilting, chlorosis, and limited necrosis. Plants are stunted.
9	Approximately 75% or more of the leaves and branches exhibit wilting, severe stunting, and necrosis with premature defoliation often resulting in plants dying.

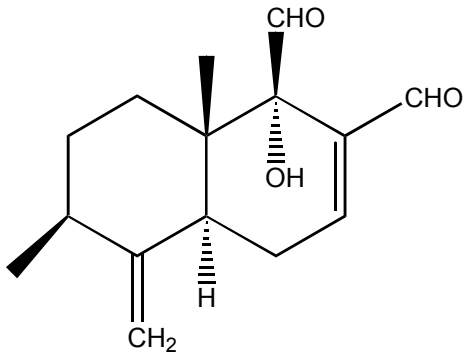
This data was recorded in tabular form for each day scored.

RESULTS AND DISCUSSION

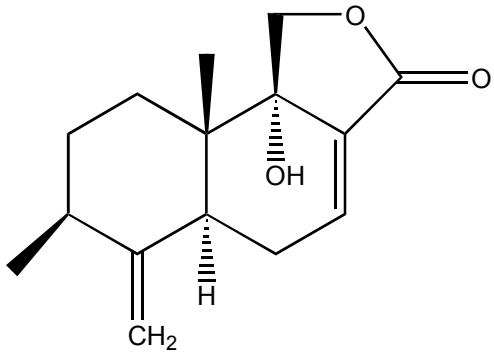
STRUCTURE DETERMINATION

Separation of *W. ugandensis* stem bark crude extract by flash chromatography yielded two pure compounds. The spectral data (^{1}H NMR, IR) and melting point data obtained for the two compounds was used for their structure determination. This was achieved by comparing with the published data (13) and that obtained from available pure standards.

The two compounds were identified as muzigadial (1) and muzigadiolide (2).



1



2

From the 15 g of the crude extract packed in the flash chromatography column, 1.030 g of muzigadial and 0.250 g of muzigadiolide were obtained. The spectral data for these two compounds is given below:

Muzigadial needles from EtOAC hexane, melting point, 124-126^o C.

^{1}H NMR (300 MHz)

σ: 9.65 (1H, s, H-12), 9.45 (1H, H-11), 7.25 (1H, t, H-7), 4.94, 4.77 (2 × 1H, 2 × br S_1 CH_2), 4.07 (1H, s, 9-OH), 263 (1H, m, H-5), 1.09 (1H, d, 3-Me), 0.88 (3H, s, 10-Me).

IR ν_{max} cm^{-1} 3460, 2950, 2850, 1715, 1670, 1630

Muzigadiolide. Needles from EtOAc-hexane, m.p. 140-144° C.

^{1}H NMR (300 MHZ)

σ: 7.19 (1H, dd, H-7), 4.92, 4.75 (2 × 1H, 2 × br s, $=CH_2$), 4.32, 4.27 (2H, 11-CH_2), 2.63 (1H, m, H-5), 1.10 (1H, d, Me), 0.75 (3H, s, 10-Me).
IR ν_{max} cm^{-1} 3400-3450, 2800, 1750, 1725, 1680, and 1635.

BIOASSAYS

Table 1 represents the assay results of the crude extracts. Crude extracts from *W. ugandensis*, *A. indica* and *T. minuta* inhibited the growth of *Fusarium oxysporum*. Crude extract from *W. ugandensis* stem bark inhibited the growth of *Alternaria passiflorae*. However the crude extract from *U. massaica* (leaves) showed no activity against *F. oxysporum* with no area of growth inhibition around the disc containing *U. massaica* crude extract.

W. ugandensis displayed a higher activity compared to *A. Indica* and *T. Minuta*. This was generally conclusive from the diameters of inhibition displayed by the crude extracts. *T. minuta* and *A. Indica* displayed a relatively similar activity against *F. Oxysporum*. Increasing amount of crude content on the disc brought about an increase in inhibition diameter. This is what is expected to take place. But the change in inhibition diameter does no seem to be proportional to the amount of crude extract on the disc. This is due to the fact that diffusion in the relatively solid media is limited as both the crude extract and the culture media were in semi-solid state.

The fractionated *W. ugandensis* leaves extract (Table 2) showed some bioactive fractions against *F. oxysporum*. This shows that the leaves are bioactive as the stem bark. Leaves are renewable and hence more preferred to other parts of plants such as the roots.

Table 1. Antifungal activity of crude extracts

Test microoganism	**Disc content (mg) Diameter (12mm)**	**Diameter of inhibition**					
		***W. ugandensis* leaves (MeOH) extract**	***W. ugandensis* Leaves (Hexane) extract**	***A. indica* leaves (MeOH) extract**	***A. indica* seeds (MeOH) extract**	***T. minuta* aerial part (MeOH) extract**	***U. massaica* leaves (MeOH) extract**
Fusarium sp.	150	23.0	16.5	15.0	18.0	16.0	No activity
	100	16.0	15.5	14.5	16.0	15.0	No activity
	50	15.5	13.0	13.0	13.5	13.0	No activity
	20	14.0	12.5	11.0	12.0	12.5	No activity
	10	12.0	11.5	10.0	9.0	10.0	No activity
	1	10.0	10.0	7.5	8.5	9.5	No activity
Alternaria passiflorae		**Stem bark ($CHCl_3$) fraction**					
	150	25.0					
	100	23.4					
	50	21.5	—	—	—	—	—
	20	19.0					
	10	15.0					
	1	11.5					

Table 2. Fractionated *W. Ugandensis* MeOH leaves extract

Test microoganism	Fractions	Disc content (mg)	Inhibition Diameter (mm)
Fusarium oxysporum	F_1	10	No growth inhibition
		1	No growth inhibition
	F_2	10	10.5
		1	8.0
	F_3	10	13.0
		1	10.0
	F_4	10	12.5
		1	10.3
	F_5	10	No growth inhibition and other fractions below F_5 had similar results.
		1	

Table 3 shows the MIC of muzigadial and mizigadiolide against *F. oxysporum*, *A. passiflorae* and *A. niger.*

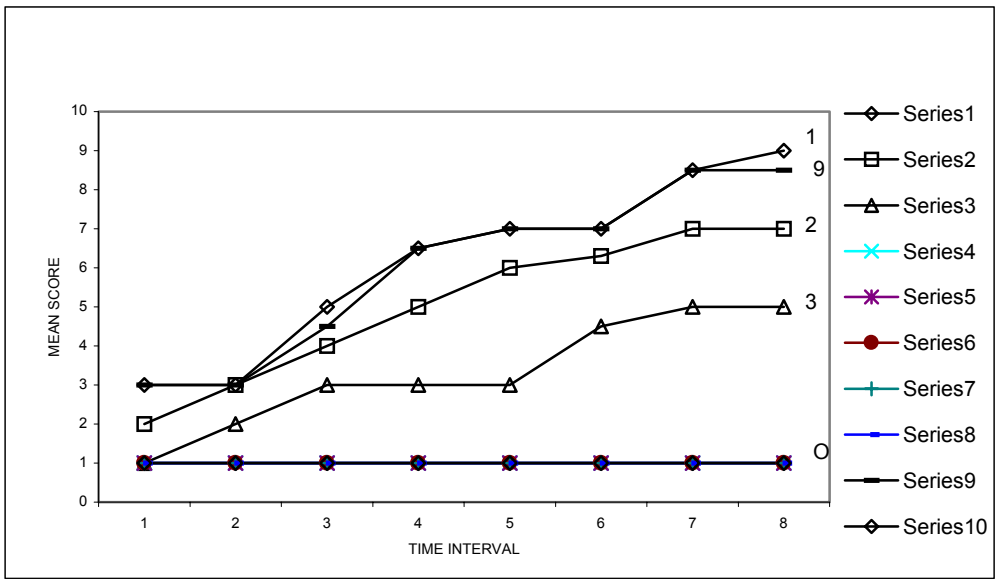
TABLE 3. Minimum Inhibitory Concentration (MIC) for muzigadial and muzigadiolide

TEST MICROORGANISM	MIC (µg/ml)	
	MUZIGADIAL	**MUZIGADIOLIDE**
Fusarium oxysporum	50	No activity
Alternaria passiflorae	>100	No activity
Aspergillus niger	5	No activity

Muzigadial was found to be active against those pathogens while muzigadiolide was not. It showed the best activity against *A. niger* with a MIC of 5µg/ml. The fact that the dialdehide functional group is responsible for the bioactivity is supported as earlier reports indicated by Kubo and Taniguchi (3).

GREEN HOUSE EXPERIMENTS

Graph 1 shows the trend of the severity data for all the days scored.



Legend

Series 1	Treatment 1, Pathogen alone
Series 2	Treatment 2, 1 mg/ml of the crude extract
Series 3	Treatment 3, 2 mg/ml ,, ,, ,,
Series 4	Treatment 4, 5 mg/ml ,, ,, ,,
Series 5	Treatment 5, 7 mg/ml ,, ,, ,,
Series 6	Treatment 6, 10 mg/ml ,, ,, ,,
Series 7	Treatment 7, 12 mg/ml ,, ,, ,,
Series 8	Treatment 8, 15 mg/ml ,, ,, ,,
Series 9	Treatment 9, Pathogen plus solvent (20 % ethanol in water)
Series 10	Treatment 10, No pathogen and no solvent; only sterilized soil

From Analysis of variance, DMRT and LSD tests, the differences among the treatments for all the days scored were significant at 1% and 5% level of significance. All the treatments except treatment 9 (pathogen plus solvent) were significantly different from control (treatment 1, pathogen alone).

Treatment 2 (1mg/ml) and treatment 3 (2mg/ml) were significantly different from the others. It showed that application of 1mg/ml and 2mg/ml concentration of crude extract was not effective in controlling the pathogen, and that concentration of 5mg/ml of the crude extract and above was effective in controlling the pathogen. Also, the solvent (20% ethanol in water) had no effect on the pathogen as plants in the pots where only the solvent was applied were equally infected as those plants in the pots where only the pathogen had been introduced (control).

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